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Genetic Evidence Linking Age-Dependent Attenuation of the 26S Proteasome with the Aging Process▽†

Ayako Tonoki,[1] Erina Kuranaga,[1,2] Takeyasu Tomioka,[1] Jun Hamazaki,[3] Shigeo Murata,[3] Keiji Tanaka,[4] and Masayuki Miura[1,2]*

Department of Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan[1]; JST, CREST, Tokyo, Japan[2]; Laboratory of Protein Metabolism, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan[3]; and Laboratory of Frontier Science, Core Technology and Research Center, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan[4]

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The intracellular accumulation of unfolded or misfolded proteins is believed to contribute to aging and age-related neurodegenerative diseases. However, the links between age-dependent proteotoxicity and cellular protein degradation systems remain poorly understood. Here, we show that 26S proteasome activity and abundance attenuate with age, which is associated with the impaired assembly of the 26S proteasome with the 19S regulatory particle (RP) and the 20S proteasome. In a genetic gain-of-function screen, we characterized *Rpn11*, which encodes a subunit of the 19S RP, as a suppressor of expanded polyglutamine-induced progressive neurodegeneration. *Rpn11* overexpression suppressed the age-related reduction of the 26S proteasome activity, resulting in the extension of flies' life spans with suppression of the age-dependent accumulation of ubiquitinated proteins. On the other hand, the loss of function of *Rpn11* caused an early onset of reduced 26S proteasome activity and a premature age-dependent accumulation of ubiquitinated proteins. It also caused a shorter life span and an enhanced neurodegenerative phenotype. Our results suggest that maintaining the 26S proteasome with age could extend the life span and suppress the age-related progression of neurodegenerative diseases.

Ubiquitin-conjugated, misfolded protein aggregates are observed in the brain during normal aging and in late-onset human neurodegenerative diseases, such as Alzheimer's, Parkinson's, and polyglutamine diseases (e.g., Huntington's disease or spinocerebellar ataxias) (9). Many of the mutations that cause dominantly inherited neurodegenerative diseases dramatically increase the amount of protein aggregates in vitro and in vivo, supporting the widely accepted hypothesis that proteotoxicity caused by the aggregates underlies the pathogenesis of many neurodegenerative diseases (32). Proteotoxicity can have many effects, including disruption of microtubule-dependent axonal transport (10), perturbation of membrane permeability (23), and impaired function of the ubiquitin-proteasome system (UPS) (1, 17). Aggregation-mediated toxicity has also been suggested in normal aging, because recent reports show that the impairment of autophagy in the central nervous system causes accumulation of ubiquitinated proteins and leads to neurodegenerative diseases (12, 21). These observations suggest that the continuous clearance of misfolded proteins through cellular degradation systems, including the UPS and autophagy, is important for preventing aggregation-mediated proteotoxicity both in age-related neurodegenerative diseases and in normal aging.

Clinical symptoms of neurodegenerative diseases generally do not appear or progress until advanced ages, not only in sporadic forms but also in inherited forms of neurodegenerative diseases (26). These observations suggest that aggregation-mediated toxicity appears in a late-onset manner both in normal aging and in neurodegenerative diseases. Furthermore, a link between the aging process and aggregation-mediated proteotoxicity has been suggested by evidence that Huntington's disease-associated proteotoxicity was ameliorated when the aging process slowed, that is, the life span extension via decreased insulin/insulin growth factor-1-like signaling in *Caenorhabditis elegans* (13, 31).

A possible mechanism for the late onset of aggregation-mediated toxicity is age-related impairment of the UPS, because loss-of-function mutations in genes encoding UPS components can enhance the cytotoxicity of protein aggregation in dominantly inherited neurodegenerative diseases (4, 5, 18). In addition, an age-related decline of proteasome activity has been observed in the tissues of humans and other mammals (8) and in aged flies (36). Considering the role of the proteasome in neuroprotection and the age dependence of most neurodegenerative diseases, the age-related decline of proteasome activity could well be a key factor both in normal aging and in the late onset and/or progression of neurodegenerative diseases. However, the mechanism underlying the age-related decline of proteasome activity remains to be elucidated, and there is no direct genetic evidence showing that the age-related decline of proteasome activity causes age-related aggregation-mediated toxicity in normal aging and in age-related neurodegenerative diseases.

Here, we studied the age-related decline of proteasome ac-

* Corresponding author. Mailing address: Department of Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81-3-5841-4860. Fax: 81-3-5841-4867. E-mail: miura@mol.f.u-tokyo.ac.jp.

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tivity by using *Drosophila melanogaster* and found age-related attenuation of the 26S proteasome activity and abundance that was associated with impaired assembly of the 26S proteasome with the 19S regulatory particle (RP) and the 20S proteasome. In a genetic gain-of-function screen, we identified *Rpn11*, which encodes one of the lid subunits in the 19S RP, as a suppressor of the age-dependent progression of a polyglutamine-induced neurodegenerative phenotype. The overexpression of *Rpn11* prevented the age-related reduction of the 26S proteasome activity, which suppressed the age-dependent accumulation of ubiquitinated proteins and extended the life span. On the other hand, the loss of function of *Rpn11* enhanced the age-related reduction of 26S proteasome activity, leading to a shorter life span, a premature age-dependent accumulation of ubiquitinated proteins, and an early onset of a neurodegenerative phenotype. Our results demonstrate for the first time that the age-related reduction of the 26S proteasome activity is a key factor in the induction of certain age-related biological changes and in the increased risk for the onset or progression of neurodegenerative diseases. Our findings imply that improving the amount and/or activity of the 26S proteasome by overexpressing a lid subunit, such as *Rpn11*, could provide an extension to the mean life span and prevent the age-dependent onset or progression of neurodegeneration.

MATERIALS AND METHODS

Fly stocks and generation of transgenic flies. Flies were raised on standard *Drosophila* medium at 25°C, and transgenic strains were generated as described previously (22). The w^{1118} strain was used as the wild-type strain. The *UAS-LacZ*, *tub-GAL80^{ts}*, *GMR-GAL4*, and *da-GAL4* (Bloomington *Drosophila* Stock Center), *UAS-MJDtr-Q78* (2), *GMR-huntingtin 120Q* (16), and *UAS-LacZIR* (20) fly strains were used in this study. The gene search (GS) system alleles were gifts from T. Aigaki. Using the temporal and regional gene expression targeting (TARGET) system, we raised flies at the restrictive temperature (18°C) before eclosion to suppress the activity of GAL4. One day after eclosion, the flies were moved to the permissive temperature (29°C) (28). For the longevity assay, more than 200 males of each genotype were collected and cultured in vials containing 20 males each. The flies were maintained at 29°C from day 2 and transferred to vials with fresh food every 3 to 4 days, and deaths were scored every day. Statistical significance was defined as a P value of <0.0001 by log-rank test.

Screening methods. To identify the suppressor for neurodegeneration-induced cell death, we adapted the P element-based GS system (33). The GS system vector contains two copies of the upstream activating sequence (UAS) enhancer adjacent to a core promoter: there is one copy near the terminal inverted repeats at each end of the vector, and each is oriented to direct transcription outward. This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS vector at various locations. For the first screen, we performed a dominant-modifier screen for heat-shocked *GAL4* (*hs-GAL4*)-mediated *Reaper* (a proapoptotic gene in *Drosophila*)-induced lethality to identify a strong suppressor of cell death. We identified 5 suppressor alleles from 1,600 alleles. Expecting that this collection of suppressor alleles would include suppressors of neurodegeneration, in the second screen, we performed a dominant-modifier screen for *GMR-GAL4*-mediated polyglutamine-induced neurodegenerative cell death. One allele, named *DANC* (*d*efender *a*gainst *n*eural *c*ell death), was identified as such a suppressor.

Immunoblotting. Whole flies or fly heads were prepared, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted as described previously (22). All samples were separated by 10% SDS-PAGE, and the blots were probed with antihemagglutinin (anti-HA) Y11 (1:500; Santa Cruz Biotechnology), mouse antiubiquitin (1:500; Stressgen Biotechnologies), anti-β-tubulin (1:500; Chemicon), anti-α2 (1:1,000) (11), or anti-Rpn11 (1:300) primary antibody and with anti-mouse immunoglobulin G-horseradish peroxidase (1:1,000; Promega) or anti-rabbit horseradish peroxidase-linked immunoglobulin G (1:1,000; Cell Signaling) secondary antibody. For the glycerol density gradient analyses, 200 μl of each fraction was precipitated with acetone and subjected to SDS-PAGE. The relative amount of each protein was determined by densitometric analysis using Image Gauge software (Fujifilm) (22). Quantitative data were obtained as the ratio of the indicated protein signal to that of the loading control for each immunoblot and were plotted as a ratio graph.

Histology and immunohistochemistry. The heads of adult flies from each genotype were fixed and embedded in Epon resin for the preparation of semithin horizontal sections (1 μm). The sections were stained with toluidine blue and examined under a light microscope as described previously (18). Immunohistochemistry treatment of eye discs from third-instar larvae was carried out as described previously (22). The following antibodies were used: rabbit anti-HA (1:500; Santa Cruz Biotechnology) and rat anti-ELAV 7E8A10 (1:20; Hybridoma Bank). Alexa Fluor 488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes and Jackson Immunoresearch and used at 1:100. All fluorescently labeled samples were examined with an LSM5 model confocal microscope (Carl Zeiss).

Proteasome activity. Whole flies or heads alone were homogenized in buffer B (25 mM Tris-HCl [pH 7.5], 2 mM ATP, 5 mM $MgCl_2$, and 1 mM dithiothreitol). Proteasome peptidase activity in the lysates was measured with a synthetic peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-AMC) (Sigma).

Glycerol density gradient analysis. The whole bodies of 70 flies of the appropriate genotypes were homogenized in buffer B. The lysates were clarified by centrifugation at 20,000 × *g*, and the lysates (1 mg of protein) were subjected to 8 to 32% (vol/vol) linear glycerol density gradient centrifugation (22 h, 83,000 × *g*). The gradient was separated into 32 fractions (11), and proteasome peptidase activity in 10 μl of each fraction was measured with Suc-LLVY-AMC activity.

Plasmid construction. The expressed sequence tag clone RE07468 was purchased from Invitrogen as full-length *Rpn11* cDNA. The sequence of the clone was confirmed, and an *Rpn11* fragment was amplified with the following PCR primers: 5′-CGG GGT ACC ACC ATG GAT CGT CTG CTA CGT CTT GGA-3′ and 5′-GCT CTA GAT CAC TTA AAG ACT ATG GTG TCC A-3′. The amplicon was then inserted into the KpnI-XbaI sites of the *pUAST* vector to generate *pUAST-Rpn11.* For the RNA interference experiment, a fragment containing the first 500 bp of the *Rpn11* open reading frame was inserted into *pUAST-R57* (a gift from R. Ueda), as previously described (22).

Anti-Rpn11 antibody. The N-terminally His_6-tagged recombinant Rpn11 (His-RPN11) protein was produced in the *Escherichia coli* strain BL21(DE3) pLysS (Novagen) using *pRSET-His-Rpn11* by incubating the bacteria for 20 h at 20°C. The recombinant His-Rpn11 protein expressed in E. coli BL21(DE3) was purified on a Ni^+ column and used to immunize rabbits (Hokudo Co.).

RT-PCR analysis. For reverse transcription-PCR (RT-PCR) analysis of the genes surrounding the *DANC* allele or of other GS system fly lines, flies expressing *DANC* or other GS system genes with *hs-GAL4* were treated or not with heat shock (twice at 37°C for 30 min, with a 30-min interval between treatments), and their total RNA was prepared 3 h later by using Trizol (Invitrogen). The RNA was reverse transcribed and subjected to PCR analysis (26 cycles) using the following primers: *Rpn11* (5′-ACT TAA AGA CTA TGG TGT CCA-3′ and 5′-TGG ATC GTC TGC TAC GTC TT-3′); *Rpn9* (5′-CGG GGT ACC ACC ATG TCC AAT CCT CAG CC-3′ and 5′-GCT CTA GAC TAA TTG GTG AGG ATT TCG GC-3′); *Rpn5* (5′-CGG GGT ACC ACC ATG GAC ACC TAT TTG TT-3′ and 5′-GCT CTA GAT TAA TCC TCG ACA GCA CAC AT-3′); *Rpn2* (5′-ATG AGT CTT ACG TCC GCC GCG-3′ and 5′-GAT GCA ACT TTT CAA CCT CGT T-3′); *α6* (5′-ATG TTT CGC AAC CAG TAC GAT AG-3′ and 5′-CTA TGG ACG CTG CTC GGT TGC AA-3′); and *GAPDH* (5′-CCA CTG CCG AGG AGG TCA ACT A-3′ and 5′-GCT CAG GGT GAT TGC GTA TGC A-3′).

RESULTS

Proteasome activity decreases and ubiquitinated proteins accumulate with age. To gain insight into the cause of the age-related proteasomal dysfunction, we assessed the peptidase activities of the proteasome with age in *Drosophila*. The peptidase activities of the proteasome in lysates of wild-type fly heads were measured by Suc-LLVY-AMC-hydrolyzing activity (chymotrypsin-like activity), which cleaves peptide bonds after hydrophobic amino acids. Lysates of wild-type fly heads showed gradually decreased proteasome activity with age (Fig. 1A). The proteasome activity remarkably decreased in flies that were 20 to 30 days posteclosion, the time when age-related symptoms, such as memory impairment, are first

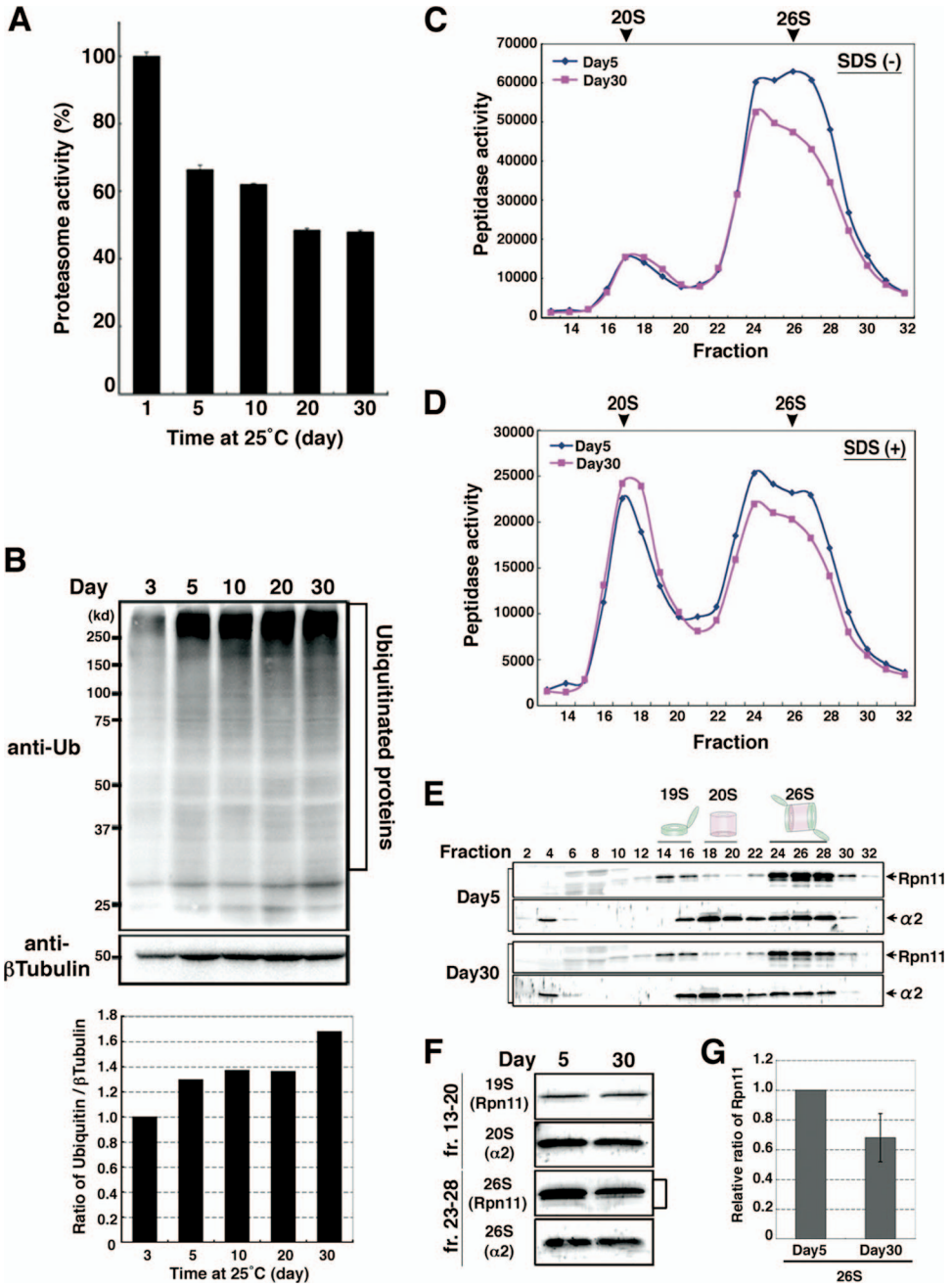

FIG. 1. The 26S proteasome activity decreases and ubiquitinated proteins accumulate with age. (A) Proteasome activity in wild-type flies decreased with age. The proteasome activity in wild-type fly heads was measured by hydrolysis of Suc-LLVY-AMC (chymotrypsin-like activity) on the indicated day posteclosion. (B) Accumulation of ubiquitinated proteins with age in wild-type flies. Wild-type fly head extracts were immunoblotted with antiubiquitin (anti-Ub) or anti-β-tubulin antibodies on the indicated day posteclosion. (C) The 26S proteasome activity in aged flies was significantly lower than that in young flies. Extracts of day 5 or day 30 flies were fractionated by glycerol density gradient centrifugation, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (D) Suc-LLVY-AMC hydrolysis activities of the same lysates shown in panel C were measured with the addition of 0.01% SDS [SDS(+)], a potent artificial activator of the 20S proteasome. The peptidase activity of the 20S proteasome did not significantly change between day 5 and day 30. (E) Immunodetection of the Rpn11 or the α2 subunit revealed a decrease in the level of this subunit with age in the 26S-containing fractions. Immunoblot analysis shows the even fractions probed with antibodies against Rpn11 and α2. (F) Fractions (fr.) 13 to 20 (19S or 20S included) or fractions 23 to 28 (26S included) shown in panel E were pooled and immunoblotted with antibodies against Rpn11 or α2. (G) The protein level of the 26S proteasome is lower in the aged fly than in the young fly. The amount of Rpn11 protein (the amount of the band indicated on panel F by a line) was determined by densitometric analysis. The graph shows the relative ratio of the amounts of Rpn11 in fractions 23 to 28 (26S included) at day 30 to that at day 5 from three individual experiments.

detectable (the normal *Drosophila* life span is 60 to 70 days) (40). This finding indicated that the reduction of proteasome activity was age dependent, as expected.

Because the proteasome maintains cellular homeostasis, we examined whether the age-related decline of proteasome activity caused the accumulation of unfolded proteins, represented by polyubiquitinated proteins. Extracts were made from wild-type fly heads or whole bodies and immunoblotted with an antiubiquitin antibody. Polyubiquitinated proteins, which showed a high-molecular-weight smear, gradually increased with the age of the flies, in both head (Fig. 1B) and whole-body extracts (see Fig. 5A). These results indicate that the reduction of proteasome activity and the accumulation of ubiquitinated proteins are observed with age.

The age-related reduction of proteasome activity results from the attenuation of the 26S proteasome. Polyubiquitinated proteins are recognized and degraded by the 26S proteasome. The 26S proteasome is a huge protein complex composed of one proteolytically active 20S proteasome and two 19S RPs, each attached to one end of the 20S proteasome (37). To compare the biochemical nature of proteasomes in young with that in aged flies, we performed an 8 to 32% glycerol density gradient centrifugation analysis and analyzed each fraction for chymotrypsin-like activity, using a peptide substrate (Suc-LLVY-AMC). The peptidase activity, particularly in the 26S proteasome-containing fractions (Fig. 1C, fractions 24 to 28) of the aged flies, was significantly lower than that of the young flies (Fig. 1C). On the other hand, the peptidase activity of the 20S proteasome, which was assayed in the presence of 0.01% SDS, a potent artificial activator of the latent 20S proteasome, did not significantly change with age (Fig. 1D).

Next, we tested the protein levels of each proteasome. Each fraction was subjected to immunoblotting with an anti-Rpn11 antibody to detect the 19S RP and the 26S proteasome or with an antibody against the α2 subunit to detect the 20S and 26S proteasomes. The immunodetection of Rpn11 and α2 revealed that the protein level of the 26S proteasome was lower in the aged fly than that in the young fly; however, the protein levels of the 20S proteasome and the 19S RP remained essentially stable with age (Fig. 1E, F, and G). These data indicate that the age-related reduction of proteasome activity results from the attenuation of the activity and amount of the assembled 26S proteasome.

Identification of *Rpn11*, a component of the 19S RP, as a suppressor of progressive neurodegeneration. Previously, we showed that the inhibition of proteasome function in flies expressing expanded polyglutamine enhances both the accumulation of ubiquitin conjugates and polyglutamine-induced neural degeneration (18). To examine whether the age-related attenuation of the 26S proteasome causes the age-dependent progression of neurodegenerative diseases, we focused on a progressive phenotype of neurodegenerative diseases. Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3, is the most common dominantly inherited progressive ataxia caused by polyglutamine expansion. Expression of a truncated form of the human MJD protein with an expanded polyglutamine domain (MJDtr-Q78) causes progressive neural degeneration in *Drosophila* (39). The expression of *MJDtr-Q78* driven by the eye-specific promoter *GMR* caused only a slight disruption of the regular, external lattice of the eye in young flies (Fig. 2A); however, the eye morphology of these flies showed progressive degeneration over time, with significant loss of pigmentation by day 15 (Fig. 2B).

To identify the suppressor of neurodegeneration-induced cell death, we adapted the P element-based GS system (33). This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS system vector, a P element-based vector with UAS enhancers, at various locations. In the course of performing a gain-of-function screen for suppressors of neural cell death, using fly alleles with the GS vector, we identified 1 of 1,600 alleles, which we called the *DANC* allele, that suppressed the progression of the *MJDtr-Q78*-induced degenerative phenotype (Fig. 2C and D, and see Materials and Methods). We used the inverse PCR method to determine the insertion site for the GS vector in the *DANC* strain; this vector can induce transcription bidirectionally from its insertion site (Fig. 2I). The PCR amplified a predicted gene, *Rpn11*, the expression level of which was elevated in a GAL4-dependent manner (Fig. 2J). Rpn11 is one lid component of the multiple subunits that make up the 19S RP. The 19S RP can be divided into two subcomplexes, known as the "base" and "lid" subcomplexes (see Fig. S2N in the supplemental material). Among the lid subunits, Rpn11 functions as a metalloprotease that cleaves the isopeptide bonds between a ubiquitinated substrate and the most proximal ubiquitin of the polyubiquitin chain (35, 41). We also examined another GS fly line, encoded by *GS13423*, in which the GS vector, inserted in the 5′ untranslated region of *Rpn11*, could induce unidirectional transcription (Fig. 2I) and drive *Rpn11* expression in a GAL4-dependent manner (Fig. 2K). *GS13423* also suppressed the progression of the *MJDtr-Q78*-induced rough eye phenotype (Fig. 2E and F). To confirm directly that *Rpn11* was responsible for the suppression of the *MJDtr-Q78*-induced phenotype in the *DANC* fly line, we generated the *UAS-Rpn11* transgenic fly lines and overexpressed *Rpn11* in the fly eye with *MJDtr-Q78*. As with the *DANC* or *GS13423* allele, the overexpression of *Rpn11* suppressed the *MJDtr-Q78*-induced progressive loss of pigmentation (Fig. 2G and H) without affecting *GAL4* expression by the overexpression of *Rpn11* or with age (see Fig. S1 in the supplemental material).

We next examined whether overexpression of *Rpn11* could suppress another model of expanded polyglutamine disease, the human Huntingtin (*Htt*) gene (16). The photoreceptor neurons in the wild-type fly eye are arranged in a series of repeating trapezoids visible as seven rhabdomeres within each ommatidium (Fig. 2L and M). When Htt peptides were expressed in the eye, although almost seven rhabdomeres were observed within each ommatidium at day 2 (Fig. 2N and R), the loss of rhabdomeres in each ommatidium was observed at day 15 (Fig. 2O and R). However, when *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly reduced (Fig. 2P, Q, and R). These data indicate that the overexpression of *Rpn11* suppresses the age-related progression of neural degeneration induced by polyglutamine.

Overexpression of *Rpn11* ameliorates the toxicity of expanded polyglutamine. A number of studies have focused on the strong linkage between the pathogenesis of neurodegenerative diseases and protein aggregation (32). We next ad-

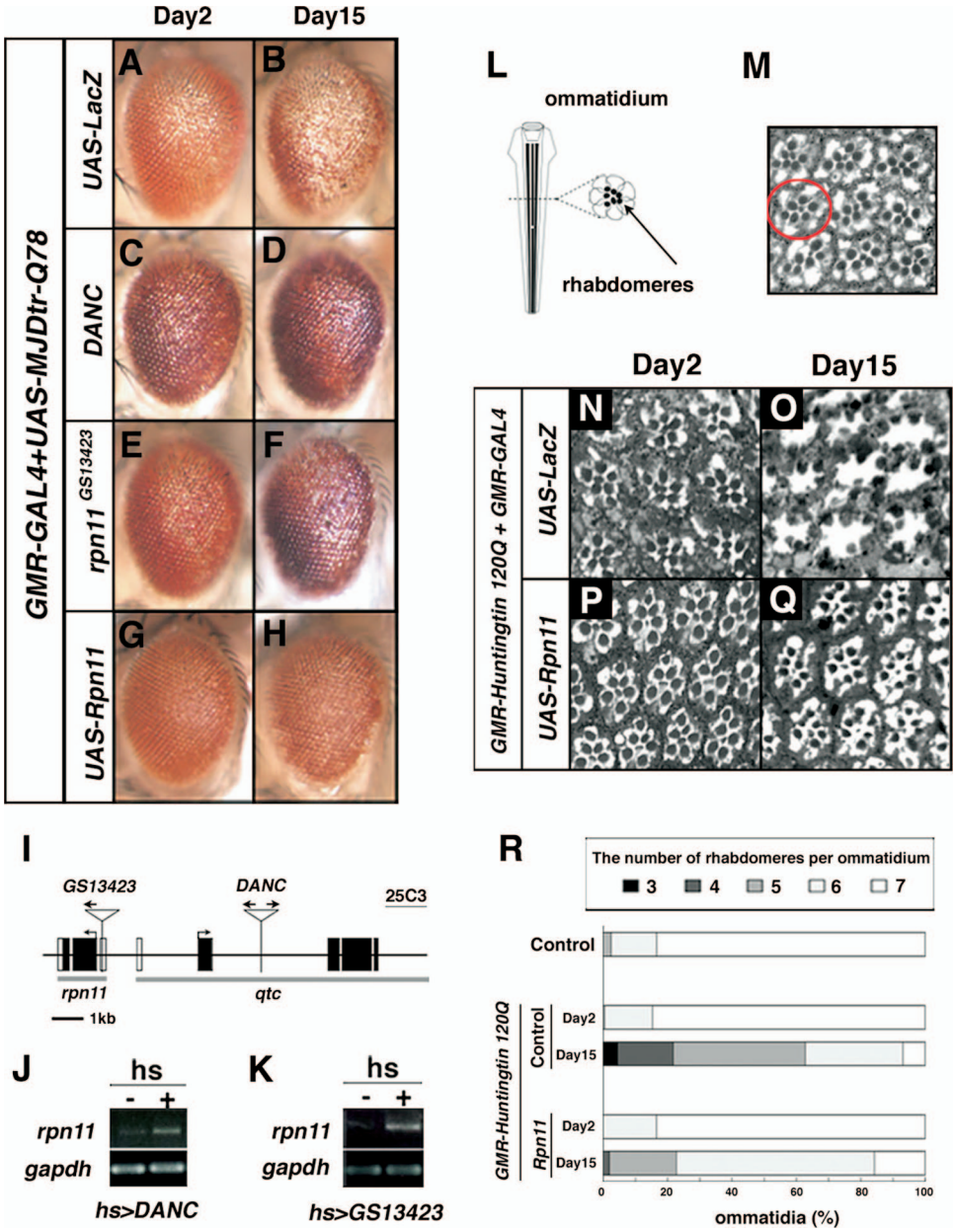

FIG. 2. Identification of *Rpn11* as a suppressor of age-related polyglutamine-induced progressive neurodegeneration. (A to H) Light photomicrographs of fly eyes expressing an expanded polyglutamine protein product of *MJDtr-Q78*, along with the indicated transgene. (A and B) Flies of genotype *w*; *GMR-GAL4*/*UAS-LacZ*; *UAS-MJDtr-Q78*/+ are shown at day 2 (A) and day 15 (B) posteclosion. These flies show eye degeneration, with a progressive loss of external pigment. (C to H) The progressive pigment loss phenotype was suppressed in flies expressing both *MJDtr-Q78* and *DANC* (C and D), *GS13423* (E and F), or *Rpn11* (G and H). The fly genotypes shown are *w*; *GMR-GAL4*/*GS13423*; *UAS-MJDtr-Q78*/+ (E and F) and *w*; *GMR-GAL4*/*UAS-Rpn11*; *UAS-MJDtr-Q78*/+ (G and H). (I) The genomic structures around the *DANC* and *GS13423* alleles are shown. (J and K) The *DANC* and *hs-GAL4* flies (J) and the *GS13423* and *hs-GAL4* flies (K) were heat shocked (hs+) or not (hs−), and the expression levels of the surrounding genes, as well as that of GAPDH, were analyzed by RT-PCR. Note that *rpn11* was upregulated in a GAL4-dependent manner. (L) Schematic of a single *Drosophila* ommatidium showing the regular trapezoidal arrangement of seven visible rhabdomeres within the photoreceptor neuron. (M) Representative semithin sections are shown of compound eyes from wild-type flies 2 days after eclosion. Normal ommatidia contain seven visible rhabdomeres at a given plane of the section. A representative single ommatidium is circled. (N to Q) Semithin sections of compound eyes from flies expressing *GMR-Huntingtin 120Q*, with *LacZ* as a control (N and O), or with *Rpn11* (P and Q). When *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly improved (N to Q). The following genotypes are shown: *w*; *UAS-LacZ*/*GMR-GAL4*; *GMR-Huntingtin120Q*/+ (N and O) and *w*; *UAS-Rpn11*/*GMR-GAL4*; *GMR-Huntingtin120Q*/+ (P and Q). (R) Quantification of the number of rhabdomeres per ommatidium. More than 100 ommatidia per eye section were counted, and at least four eyes were sectioned for each fly line. The mean number of rhabdomeres per ommatidium ± SD for the control flies was 6.7 ± 0.5; that for the *Htt* plus control *LacZ* flies on day 15 was 5.2 ± 0.9; and that for the *Htt* plus *Rpn11* flies on day 15 was 5.9 ± 0.6. Differences between the *Htt* plus control *LacZ* flies on day 15 and the *Htt* plus *Rpn11* flies on day 15 are significant: $P < 0.0001$ (Student's *t* test).

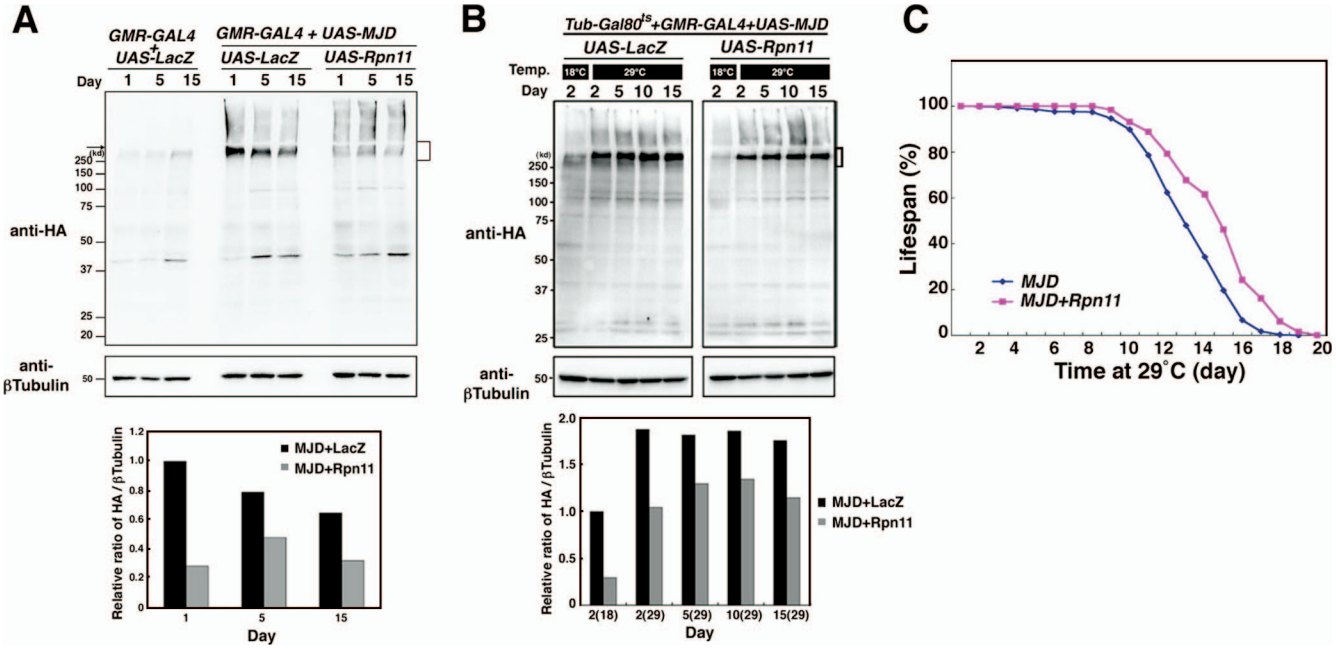

FIG. 3. Overexpression of *Rpn11* ameliorates the toxicity caused by expanded polyglutamine. (A) The coexpression of *Rpn11* with *MJDtr-Q78* (MJD+Rpn11) significantly decreased the polyglutamine-induced aggregation. The expanded polyglutamine protein in flies ran as an SDS-insoluble complex in the stacking gel and at the top of the separating gel and was detected by immunoblot analysis with an anti-HA antibody, with which *MJDtr-Q78* was tagged. Fly heads from each line of the indicated genotype were subjected to immunoblotting with an anti-HA antibody and an anti-β-tubulin antibody as a loading control. Fly genotypes were *w*; *GMR-GAL4*/*UAS-LacZ*, *w*; *GMR-GAL4*/*UAS-LacZ*; *UAS-MJDtr-Q78*/+ and *w*; *GMR-GAL4*/*UAS-Rpn11*; *UAS-MJDtr-Q78*/+. (B) The overexpression of *Rpn11* after eclosion suppressed the aggregation induced by the eye-specific expression of the polyglutamine protein. Fly genotypes were *w*; *tub-GAL80^{ts}*/*UAS-LacZ*; *GMR-GAL4*/*UAS-MJDtr-Q78*/+ and *w*; *tub-GAL80^{ts}*/*UAS-Rpn11*; *GMR-GAL4*,*UAS-MJDtr-Q78*/+. Graphs show the ratio of HA to β-tubulin (A and B). The relative amounts of each protein were determined by densitometric analysis. For the anti-HA antibody, the band indicated on the figure by a line was analyzed. (C) The coexpression of *Rpn11* and *MJDtr-Q78* in adult flies partially suppressed the short life span of *MJDtr-Q78* flies (the mean life span ± SD for *MJD* flies was 12.3 ± 0.1 days, $n = 640$; that for *MJD* plus *Rpn11* flies was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$). Fly genotypes were *w*; *tub-GAL80^{ts}*/+; *da-GAL4*/*UAS-MJDtr-Q78* and *w*; *tub-GAL80^{ts}*/*UAS-Rpn11*; *da-GAL4*/*UAS-MJDtr-Q78*.

dressed whether the suppression of polyglutamine-induced neural degeneration by *Rpn11* was accompanied by a change in polyglutamine protein aggregate formation. To do this, we performed an immunoblot analysis of HA-tagged MJDtr-Q78. The expanded polyglutamine protein ran as an SDS-insoluble complex at the top of the separating gel and in the stacking gel; however, the amount of SDS-insoluble complex was significantly decreased in flies expressing *MJDtr-Q78* with *Rpn11* (Fig. 3A).

We next determined whether *Rpn11* expression is required during development or only during adulthood to suppress the polyglutamine protein aggregation. We adapted the TARGET system to control gene expression temporally (28). In this system, the GAL4-UAS system is conditionally regulated by a temperature-sensitive allele of *GAL80*. At a restrictive temperature (18°C), the activity of GAL4 is repressed, whereas this repression is relieved by a temperature shift to the permissive temperature (29°C). When *MJDtr-Q78* was expressed after eclosion, using the TARGET system, the SDS-insoluble complex was clearly detected from day 2 to day 15. However, when *Rpn11* was coexpressed after eclosion, the SDS-insoluble complex was significantly reduced (Fig. 3B).

To address whether *Rpn11* suppressed neurodegeneration by modulating the toxicity of the aggregated polyglutamine proteins, we assessed the life span of *MJDtr-Q78* flies overexpressing *Rpn11*. The coexpression of *Rpn11* with *MJDtr-Q78* during adulthood in the whole bodies of adult flies partially suppressed the *MJDtr-Q78*-induced shortening of the life span (the mean life span ± standard deviation [SD] for flies expressing *MJDtr-Q78* alone was 12.3 ± 0.1 days, $n = 640$; that for flies expressing *MJDtr-Q78* plus *Rpn11* was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$) (Fig. 3C). Thus, the overexpression of *Rpn11*, even occurring only in the adult flies, reduced the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins.

Other lid subunits, but not subunits of the base or the 20S proteasome, may suppress the polyglutamine-induced phenotype. Next, we assessed whether the suppressive effect of *Rpn11* on the progression of the polyglutamine-induced neurodegenerative phenotype was unique among proteasome components. For this experiment, we used GS fly lines that expressed different components of the 26S proteasome in a GAL4-dependent manner (see Fig. S2K to M in the supplemental material). We found that the overexpression of several lid subunits (see Fig. S2N in the supplemental material), *rpn9* (see Fig. S2C and D in the supplemental material) and *rpn5* (see Fig. S2E and F in the supplemental material), as well as *rpn11*, suppressed the progression of the polyglutamine-induced neurodegenerative phenotype. However, neither the *rpn2* base subunit (see Fig. S2G and H in the supplemental material) nor the 20S α*6*

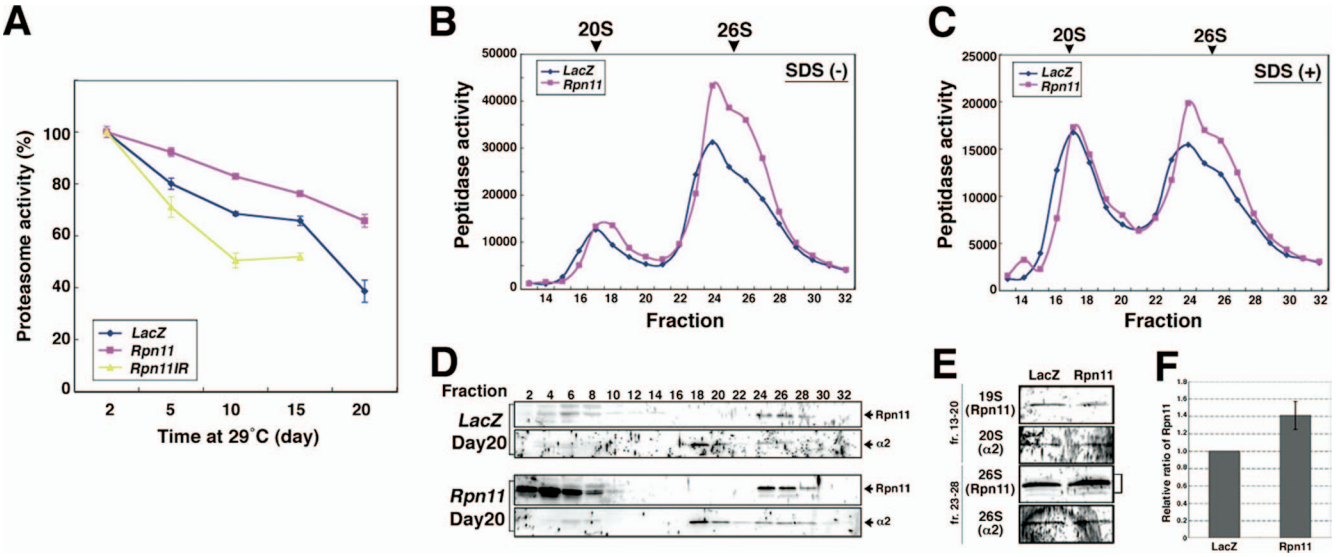

FIG. 4. Overexpression of *Rpn11* suppresses the age-related reduction of the 26S proteasome activity. (A) Proteasome activity in whole *Drosophila* with overexpression or knockdown of *rpn11* after eclosion. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4/+*, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/+*, and *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-Rpn11IR*. (B) The peptidase activity in the 26S proteasome fraction of 20-day-old flies expressing *Rpn11* was significantly higher than that of control flies. Extracts of flies overexpressing *LacZ* or *Rpn11* at day 20 were fractionated, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (C) The peptidase activity of the 20S proteasome in flies expressing *LacZ* was not different from that of flies expressing *Rpn11*. Suc-LLVY-AMC hydrolysis activities of the lysates used for panel B were measured with the addition of 0.01% SDS [SDS(+)]. (D) The amount of Rpn11 in the 26S proteasome fraction was significantly greater in flies overexpressing *Rpn11* at day 20 than that in control flies. Immunoblot analysis for the even fractions shown in panel B was performed with anti-Rpn11 or anti-α2 antibody. (E) Fractions (fr.) 13 to 20 or fractions 23 to 28 in panel D were pooled and immunoblotted with anti-Rpn11 or anti-α2. (F) The amount of Rpn11 in the 26S fractions was significantly greater in flies overexpressing *Rpn11*. The amount of Rpn11 protein (the amount of the band shown in panel E by a line) was determined by densitometric analysis. The graph shows the ratio of the amount of Rpn11 in fractions 23 to 28 (26S included) in flies expressing *Rpn11* at day20 to the amount of Rpn11 in fractions 23 to 28 in flies expressing *LacZ* at day 20 from three individual experiments.

subunit (see Fig. S2I and J in the supplemental material) could do so. Whereas GS system-based gene overexpression might induce expression of genes that are not closed to the GS system insertion site, these results suggest that the additional expression of at least some lid subunits suppresses the progression of expanded polyglutamine-induced degeneration, which implies that they might also have the ability to suppress the age-dependent impairment of proteasome activity.

The age-related reduction of proteasome activity is suppressed by the overexpression of *Rpn11*. To assess whether the ameliorative effect of *Rpn11* on the progression of expanded polyglutamine-induced degeneration was caused by preventing the age-related attenuation of the proteasome, we analyzed proteasome activity in flies expressing *Rpn11* with age. We used the TARGET system to express *Rpn11* only after eclosion, because *Rpn11* expression only in the adult was sufficient to reduce the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins (Fig. 3B and C) and because the additional expression of proteasome subunits during development might have affected development and reproduction. With the TARGET system, *Rpn11* expression was prevented when flies were raised at 18°C and was permitted when the flies were switched to 29°C at 1 day posteclosion (28). The overexpression of *Rpn11* after eclosion significantly prevented the age-related reduction of proteasome activity seen in the *LacZ* control, especially at day 20 (Fig. 4A).

To examine how the overexpression of *Rpn11* prevented the age-related reduction of proteasome activity, extracts of 20-day-old flies expressing *Rpn11* were fractionated by 8 to 32% glycerol density gradient centrifugation. When *Rpn11* was overexpressed, the peptidase activity in the 26S proteasome-containing fraction of 20-day-old flies was higher than that in the control flies (Fig. 4B). However, the peptidase activity of the 20S proteasome did not significantly change (Fig. 4C). The immunodetection of Rpn11 or α2 revealed that the amount of Rpn11 in the 26S proteasome fractions was significantly greater in flies overexpressing *Rpn11* (Fig. 4D, E, and F), even though the protein levels of the 19S RP and the 20S proteasome showed almost no changes (Fig. 4E). These results suggest that the ectopic expression of *Rpn11* after eclosion helped to maintain the activity and amount of the 26S proteasome with age.

Overexpression of *Rpn11* suppresses the age-related accumulation of ubiquitinated proteins and extends the life span. Next, to examine whether the maintenance of the 26S proteasome by the ectopic expression of *Rpn11* could also suppress the age-dependent accumulation of misfolded proteins, as it did with the accumulation of polyglutamine proteins (Fig. 3A and B), fly extracts were immunoblotted with the antiubiquitin antibody. Although the amount of polyubiquitinated proteins gradually increased with age in the control flies, this age-dependent accumulation was significantly suppressed when *Rpn11* was overexpressed after eclosion (Fig. 5A). If the age-dependent accumulation of polyubiquitinated proteins is associated with proteotoxicity in vivo, it is possible that the age-

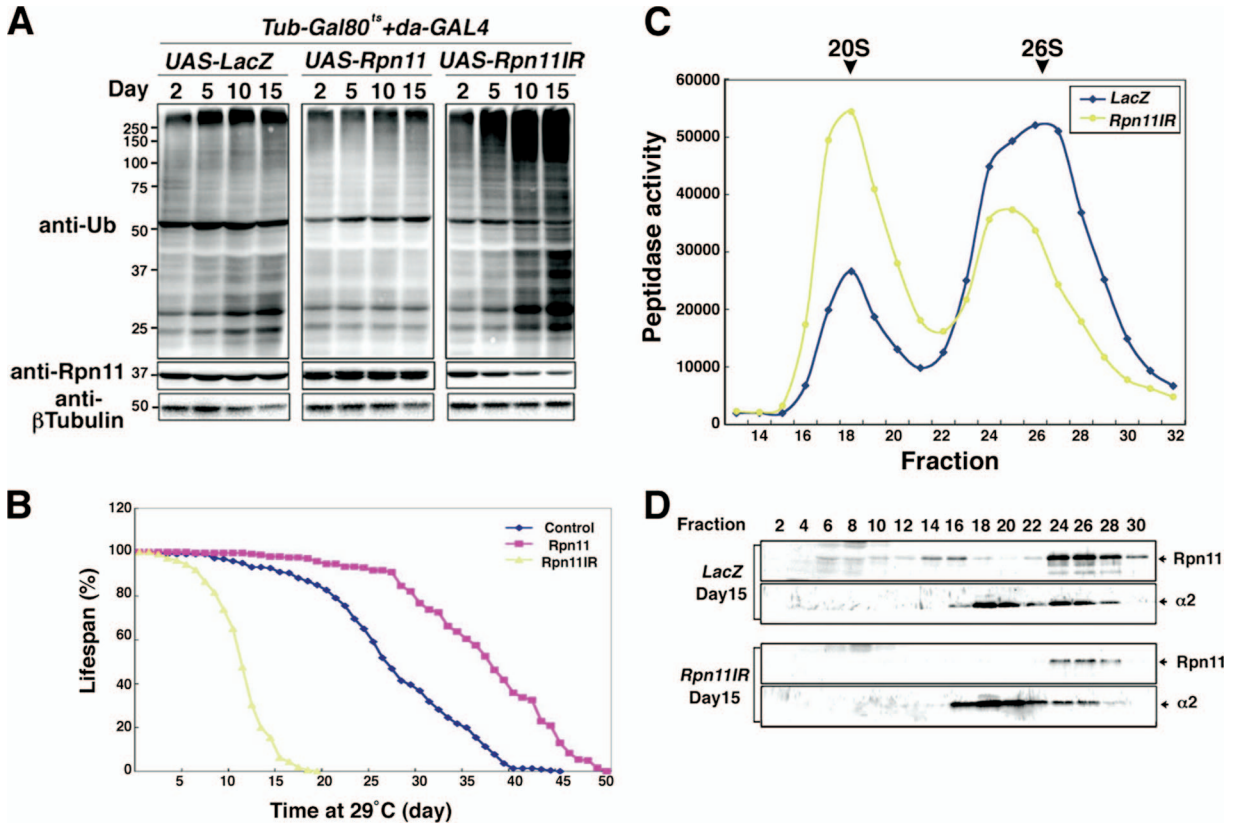

FIG. 5. *Rpn11* is required to suppress the age-dependent accumulation of ubiquitinated proteins and to extend the life span. (A) The accumulation of ubiquitinated proteins with age in the adult body was suppressed by the overexpression of *Rpn11* after eclosion and enhanced by the knockdown of *rpn11*. Whole flies from each line of the indicated genotype at the indicated day posteclosion were subjected to immunoblotting with antiubiquitin (anti-Ub), anti-Rpn11, and anti-β-tubulin antibodies. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4*/+, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4*/+, and *w*; *tub-GAL80^{ts}*/+; *da-GAL4/UAS-Rpn11IR*. Numbers at left are molecular masses (in kDa). (B) The overexpression of *Rpn11* significantly extended the life span compared with that of control flies (the mean life span ± SD of control flies was 27.2 ± 0.56 days, $n = 220$; and for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, $n = 240$; log-rank test, $P < 0.0001$). On the other hand, the ubiquitous expression of *Rpn11IR* in adult flies reduced their life span (the mean life span ± SD for control flies was 27.2 ± 0.56 days, $n = 220$; and for the *rpn11* knockdown line was 11.1 ± 0.18, $n = 320$; log-rank test, $P < 0.0001$). Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-GFP*; *da-GAL4*/+, *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4*/+, and *w*; *tub-GAL80^{ts}*/+; *da-GAL4/UAS-Rpn11IR*. (C) Knockdown of *rpn11* led to a decrease in the 26S proteasome activity. Extracts of flies expressing *LacZ* or *Rpn11IR* ubiquitously for 15 days after eclosion were fractionated by 8 to 32% glycerol gradient centrifugation. Genotypes shown are *w*; *tub-GAL80^{ts}/UAS-LacZ*; *da-GAL4*/+ and *w*; *tub-GAL80^{ts}*/+; *da-GAL4/UAS-Rpn11IR*. (D) Immunodetection of Rpn11 or α2 revealed that the knockdown of *rpn11* inhibited the assembly of the 26S proteasome. The levels of Rpn11 and α2 in the fractions containing the 26S proteasome (fractions 24 to 30) were significantly decreased. Immunoblot analysis was performed for each fraction, using antibodies against Rpn11 or α2.

dependent accumulation of polyubiquitinated proteins affects the life span. Therefore, we compared the life span of control flies with that of flies expressing *Rpn11* after eclosion. The overexpression of *Rpn11* during adulthood significantly extended the mean life span compared with that of control flies (mean life span ± SD for control flies was 27.2 ± 0.56 days, $n = 220$; life span for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, $n = 240$; log-rank test, $P < 0.0001$) (Fig. 5B). Thus, the overexpression of *Rpn11* suppresses the age-dependent accumulation of ubiquitinated proteins and extends the mean life span in flies. These results imply that the maintenance of the 26S proteasome with age prevents the age-related aggregation-mediated toxicity.

Knocking down of *rpn11* enhances the age-related accumulation of ubiquitinated proteins and shortens the life span, accompanied by reduced 26S proteasome. To examine whether impairment of the 26S proteasome activity enhanced the age-related aggregation-mediated toxicity, we assessed the accumulation of ubiquitinated proteins and the life span in the *rpn11* knockdown flies. We generated transgenic flies bearing an inverted repeat (IR) fragment of the *Rpn11* cDNA in the *pUAST* vector (*Rpn11IR*) that would specifically inhibit *rpn11* expression in a GAL4-dependent manner via a mechanism of RNA interference. When *rpn11* was knocked down in whole-body extracts after eclosion by an *Rpn11IR*, the age-dependent reduction of proteasome activity was enhanced (Fig. 4A), along with the attenuation of the activity and amount of the 26S proteasome (Fig. 5C and D), suggesting that the assembly of the 26S proteasome was impaired. In the *rpn11* knockdown flies, the accumulation of ubiquitinated proteins was clearly enhanced with age (Fig. 5A). In addition, the mean life span in the *rpn11* knockdown flies was severely impaired (mean life span ± SD for control flies was 27.2 ± 0.56 days, $n = 220$; and for the *rpn11*

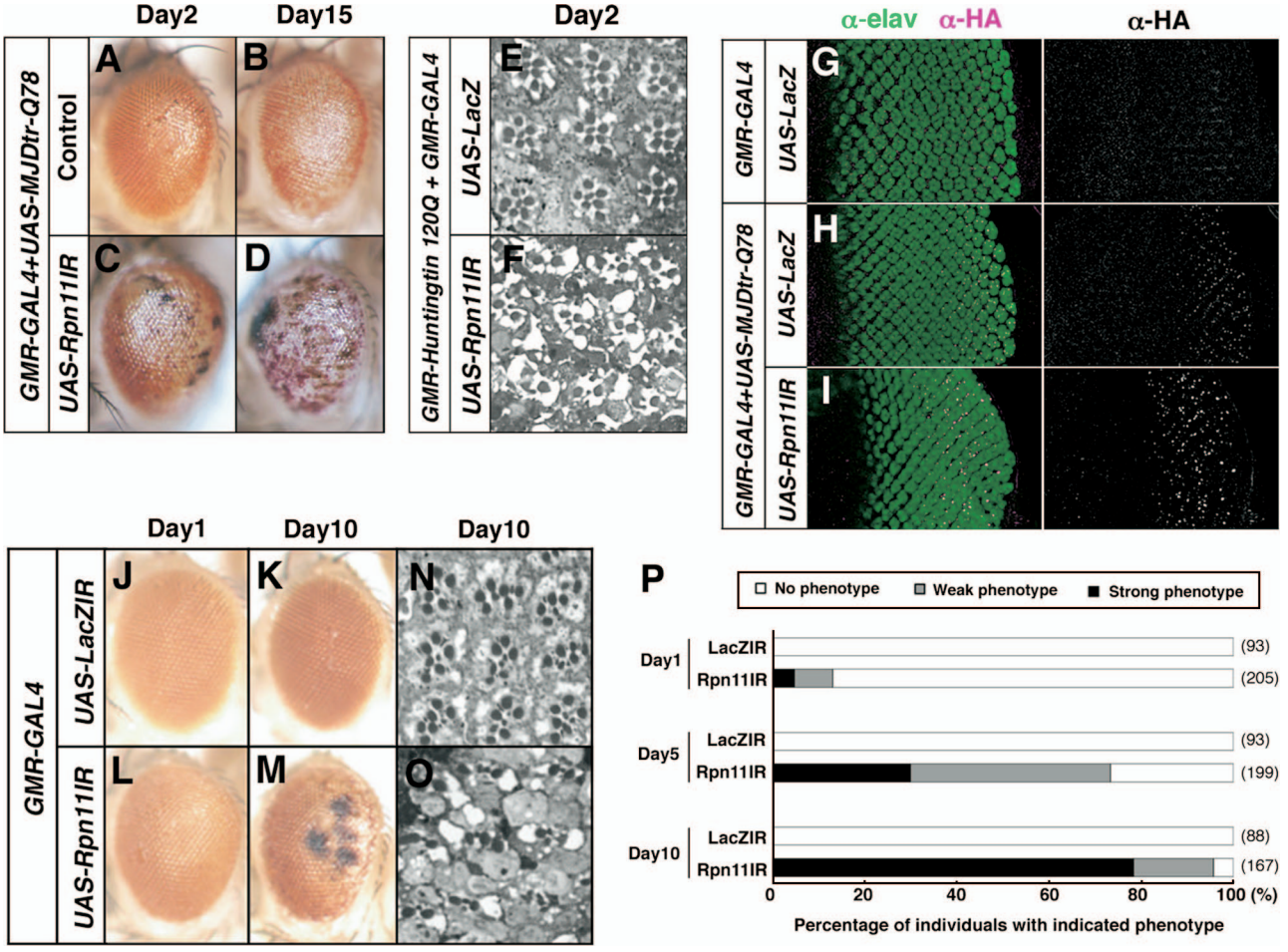

FIG. 6. Knocking down *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. (A to F) Knocking down *rpn11* clearly enhances the phenotype of polyglutamine-induced neurodegeneration. Light microscopy (A to D) and semithin-section (E and F) images of the compound eyes are shown. The following genotypes are shown: *w*; *GMR-GAL4*/*UAS-LacZ*; *UAS-MJDtr-Q78*/+ (A, B) and *w*; *GMR-GAL4*/+; *UAS-MJDtr-Q78*/*UAS-Rpn11IR* (C and D), *w*; *UAS-LacZ*/*GMR-GAL4*; *GMR-Huntingtin120Q*/+ (E), and *w*; *GMR-GAL4*/+; *GMR-Huntingtin120Q*/*UAS-Rpn11IR* (F). (G to I) Polyglutamine aggregation was significantly enhanced when *rpn11* was knocked down. Eye imaginal discs of wandering third-instar larvae were immunostained with anti-ELAV (green) and anti-HA (magenta), which *MJDtr-Q78* was tagged with. ELAV is expressed in all photoreceptor neurons. The following genotypes are shown: *w*; *GMR-GAL4*/*UAS-LacZ* (G), *w*; *GMR-GAL4*/*UAS-LacZ*; *UAS-MJDtr-Q78*/+ (H), and *w*; *GMR-GAL4*/+; *UAS-MJDtr-Q78*/*UAS-Rpn11IR* (I). (J to O) Knocking down *rpn11* caused the severe neural degeneration. Light microscopy (J to M) and semithin-section (N and O) images of fly eyes expressing *Rpn11IR* (L, M, and O) or control protein (J, K, and N) are shown. The following genotypes are shown: *w*; *GMR-GAL4*/*UAS-LacZIR* at day 1 (J) and day 10 (K and N), respectively, and *w*; *GMR-GAL4*/*UAS-Rpn11IR* at day 1 (L) and day 10 (M and O), respectively. (P) The *Rpn11IR*-induced neural degeneration is progressive. Quantification of the appearance of black dots on the surface of the eye with age is shown. A weak or strong phenotype is categorized as the appearance of one black dot or the appearance of more than two black dots, respectively. Numbers of individuals counted are shown at the right. Progressive phenotypes are seen in flies in which *rpn11* was knocked down.

knockdown fly line, life span was 11.1 ± 0.18 days, $n = 320$; log-rank test, $P < 0.0001$) (Fig. 5B).

Knocking down of *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. Next, we examined whether the impairment of the 26S proteasome activity enhances the toxicity of expanded polyglutamine. When *rpn11* was knocked down in the *MJDtr-Q78*-expressing fly eye, the *MJDtr-Q78*-induced progressive loss of pigmentation was clearly enhanced (Fig. 6A to D). In addition, when *Rpn11IR* was coexpressed with *Htt*, the loss of rhabdomeres was severe even at day 2 (Fig. 6E and F).

To determine whether knocking down of *rpn11* enhanced polyglutamine aggregation, we examined polyglutamine aggregation formation in developing eye discs of larvae expressing polyglutamine. Polyglutamine aggregation was observed in eye discs of larvae expressing expanded polyglutamine protein, which was detected with antibody to the HA tag (Fig. 6H). When *rpn11* was knocked down, polyglutamine aggregation was significantly enhanced (Fig. 6I).

Next, we examined whether the impairment of the 26S proteasome activity leads to a neurodegenerative phenotype. While the eye-specific knockdown of *rpn11* resulted in an almost normal phenotype on day 1 after eclosion (Fig. 6L and P), by day 10 after eclosion, black dots progressively appeared on the surface of the eye with age (Fig. 6M and P). In addition,

retinal sections showed that knocking down *rpn11* caused the severe loss of rhabdomeres (Fig. 6N and O). These data indicate that impairment of the 26S proteasome leads to retinal degeneration in otherwise wild-type individuals and to early onset of polyglutamine-induced defects.

DISCUSSION

Although aggregation-mediated toxicity is involved in both normal aging and neurodegenerative disease, the reason that aggregation-mediated toxicity emerges late in life has not been elucidated. Our results demonstrate for the first time that an age-related reduction of the 26S proteasome activity affects longevity and could underlie the induction of certain effects of aging and the age-dependent increased risk of the onset and/or progression of neurodegeneration in vivo.

In this study, we demonstrated that the age-related reduction of proteasome activity results from the attenuation of both the activity and amount of the 26S proteasome, which is associated with impaired assembly of the 26S proteasome from the 19S RP and the 20S proteasome in vivo. Age-related proteasome dysfunction has been reported in studies on humans, other mammals, and flies (3, 19). Thus, the age-related reduction of proteasome activity is evolutionarily conserved. Importantly, our data indicated that the reduction of the 26S proteasome activity with age might result from a defect in its assembly. This indication is supported by our data that the lysates from the young flies showed two peaks of peptidase activity (Fig. 1C, fractions 24 and 26) among the 26S proteasome fractions, which represented the 26S proteasome with one or two RP caps, but aged flies showed only one peak (Fig. 1C, fraction 24), which indicates that the 26S proteasome with one cap was the predominant form (Fig. 1C).

The free 20S proteasome is almost inactive and cannot degrade multiubiquitinated proteins because the pores leading into the catalytic chamber are closed. The opening of these gates is triggered by the 19S RP attached to one end of the 20S proteasome (6). The assembly of the 26S proteasome is known to be ATP dependent, and a recent report indicates that ATP binding is sufficient to promote the assembly of the 26S proteasome from the 19S RP and the 20S proteasome (25). That report's data indicate that no additional assembly factors are required in vitro (25). Therefore, we at first suspected that an age-associated decline in ATP levels caused the disassembly of the 26S proteasome. However, no decline in ATP levels could be detected, even in flies that were 30 days posteclosion (data not shown), despite the obvious decline of proteasome activity (Fig. 1A). Thus, specific regulators besides ATP may affect the assembly of the 26S proteasome in vivo.

Previous reports have identified extraproteasomal proteins that promote the assembly of the 26S proteasome. For example, Hsp90, an ATP-regulated chaperone, promotes the assembly and maintains the stability of the 26S proteasome in *Saccharomyces cerevisiae* (14). Ecm29 has also been identified as a stoichiometric binding partner for the yeast 26S proteasome and is proposed to enhance its stability by binding to both the 20S proteasome and the 19S RP (24). It is possible that these specific regulators of the assembly or stability of the 26S proteasome are affected by age and cause the age-related decline of the 26S proteasome activity.

Our results demonstrate that an age-related reduction of the 26S proteasome activity could be key to the age-related accumulation of misfolded or unfolded proteins and the duration of the life span. This idea that the homeostasis of general protein folding is important during aging is supported by recent findings for the role of chaperones in promoting longevity in *C. elegans* and other organisms. For example, overexpression of either the HSP70 or HSP16 chaperone, which resists protein misfolding, increases the life span (38, 42), whereas reduced chaperone expression by *heat shock factor-1* (*hsf-1*) knockdown shortens the life span (13). Although the direct effects of the proteasome function on life span have been less well explored, a recent report suggests that AIRAP (arsenic-inducible proteasomal 19S regulatory particle-associated protein), which associates tightly with the 19S proteasome, is involved in regulating the life span. The report showed that *C. elegans* lacking *aip-1*, a homologue of mammalian *AIRAP*, exhibit a shortened life span (43). Taken together with our findings, these results support our idea that maintenance of the 26S proteasome activity with age should promote longevity, implying that the age-related decline of the 26S proteasome activity is an important element in determining longevity.

Although genetic variants, behaviors, and environmental factors have been associated with increased risk of disease (27), our genetic evidence indicates that the age-related decline of the 26S proteasome activity could be a key risk factor for the progression or late onset of neurodegenerative diseases, because the overexpression of *Rpn11* suppressed the progression of polyglutamine-induced neurodegeneration with the maintenance of the 26S proteasome activity during aging. It is possible that Rpn11 functions as a metalloprotease and suppresses the polyglutamine-induced neurodegeneration by upregulation of the deubiquitinating activity. However, because it is reported that Rpn11 could not work by itself (35, 41), it is unlikely that Rpn11 functions as a metalloprotease. In fact, we measured the deubiquitinating activity in flies overexpressing *Rpn11*, but it did not change significantly compared with that of the control flies (data not shown).

Although further study is required to determine the precise details of how *Rpn11* suppresses the age-related impairment of proteasome activity, our data indicate that this mechanism could involve the promotion of 26S proteasome assembly. We revealed that GS system-based overexpression of some lid subunits, such as *Rpn11*, *-9*, and *-5*, suppressed the progression of polyglutamine-induced neurodegenerative phenotypes (see Fig. S2 in the supplemental material). Some reports indicate the existence of a core interaction cluster composed of the lid components Rpn11, -9, -5, and -8 (7, 15). It is possible that the expression of any given lid subunit could promote assembly of the lid complex, which could conceivably drive the assembly of the 26S proteasome by increasing the pool of 19S RPs. Although it is conceivable that the overexpression of *Rpn11* could lead to the upregulation of other proteasome components, our quantitative PCR data showed that overexpression of *Rpn11* did not affect the expression of other proteasome components or of the autophagy-related gene *atg5* (see Fig. S3 in the sup-

plemental material). These lines of evidence suggest that Rpn11 could promote the assembly of the 26S proteasome from free subunits without the upregulation of other lid subunits.

We have examined age-related attenuation of the 26S proteasome as one component in a process of protein degradation. Because the network of protein homeostasis is a complex process, it is possible that chaperone and autophagy could also gradually decrease and play an important role in the late onset or progression of neurodegenerative diseases (29, 30). Given the importance of maintaining the 26S proteasome activity, the mechanism for the late onset or progression of neurodegenerative diseases may be revealed by future studies of specific regulators of the 26S proteasome, for example, the 19S lid subunits such as Rpn11, some unidentified lid assembler, or a specific assembler for the 26S proteasome. This knowledge might be translated into the development of valuable therapeutic tools for the treatment of progressive age-related neurodegenerative diseases, including polyglutamine-induced disease.

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REFERENCES

1. **Bence, N. F., R. M. Sampat, and R. R. Kopito.** 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. Science **292:**1552–1555.
2. **Bonini, N. M.** 2002. Chaperoning brain degeneration. Proc. Natl. Acad. Sci. USA **99**(Suppl. 4)**:**16407–16411.
3. **Chondrogianni, N., and E. S. Gonos.** 2005. Proteasome dysfunction in mammalian aging: steps and factors involved. Exp. Gerontol. **40:**931–938.
4. **Cummings, C. J., E. Reinstein, Y. Sun, B. Antalffy, Y. Jiang, A. Ciechanover, H. T. Orr, A. L. Beaudet, and H. Y. Zoghbi.** 1999. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Neuron **24:**879–892.
5. **Fernandez-Funez, P., M. L. Nino-Rosales, B. de Gouyon, W. C. She, J. M. Luchak, P. Martinez, E. Turiegano, J. Benito, M. Capovilla, P. J. Skinner, A. McCall, I. Canal, H. T. Orr, H. Y. Zoghbi, and J. Botas.** 2000. Identification of genes that modify ataxin-1-induced neurodegeneration. Nature **408:**101–106.
6. **Forster, A., and C. P. Hill.** 2003. Proteasome degradation: enter the substrate. Trends Cell Biol. **13:**550–553.
7. **Fu, H., N. Reis, Y. Lee, M. H. Glickman, and R. D. Vierstra.** 2001. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. **20:**7096–7107.
8. **Gaczynska, M., P. A. Osmulski, and W. F. Ward.** 2001. Caretaker or undertaker? The role of the proteasome in aging. Mech. Ageing Dev. **122:**235–254.
9. **Gray, D. A., M. Tsirigotis, and J. Woulfe.** 2003. Ubiquitin, proteasomes, and the aging brain. Sci. Aging Knowledge Environ. **2003:**RE6.
10. **Gunawardena, S., L. S. Her, R. G. Brusch, R. A. Laymon, I. R. Niesman, B. Gordesky-Gold, L. Sintasath, N. M. Bonini, and L. S. Goldstein.** 2003. Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*. Neuron **40:**25–40.
11. **Hamazaki, J., S. Iemura, T. Natsume, H. Yashiroda, K. Tanaka, and S. Murata.** 2006. A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes. EMBO J. **25:**4524–4536.
12. **Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima.** 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature **441:**885–889.
13. **Hsu, A. L., C. T. Murphy, and C. Kenyon.** 2003. Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science **300:**1142–1145.
14. **Imai, J., M. Maruya, H. Yashiroda, I. Yahara, and K. Tanaka.** 2003. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. EMBO J. **22:**3557–3567.
15. **Isono, E., N. Saito, N. Kamata, Y. Saeki, and A. Toh-E.** 2005. Functional analysis of Rpn6p, a lid component of the 26 S proteasome, using temperature-sensitive rpn6 mutants of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **280:**6537–6547.
16. **Jackson, G. R., I. Salecker, X. Dong, X. Yao, N. Arnheim, P. W. Faber, M. E. MacDonald, and S. L. Zipursky.** 1998. Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. Neuron **21:**633–642.
17. **Jana, N. R., E. A. Zemskov, and N. Wang Gh.** 2001. Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. Hum. Mol. Genet. **10:**1049–1059.
18. **Kanuka, H., E. Kuranaga, T. Hiratou, T. Igaki, B. Nelson, H. Okano, and M. Miura.** 2003. Cytosol-endoplasmic reticulum interplay by Sec61alpha translocon in polyglutamine-mediated neurotoxicity in *Drosophila*. Proc. Natl. Acad. Sci. USA **100:**11723–11728.
19. **Keller, J. N., J. Gee, and Q. Ding.** 2002. The proteasome in brain aging. Ageing Res. Rev. **1:**279–293.
20. **Kennerdell, J. R., and R. W. Carthew.** 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. Nat. Biotechnol. **18:**896–898.
21. **Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka.** 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature **441:**880–884.
22. **Kuranaga, E., H. Kanuka, A. Tonoki, K. Takemoto, T. Tomioka, M. Kobayashi, S. Hayashi, and M. Miura.** 2006. *Drosophila* IKK-related kinase regulates nonapoptotic function of caspases via degradation of IAPs. Cell **126:**583–596.
23. **Lashuel, H. A., D. Hartley, B. M. Petre, T. Walz, and P. T. Lansbury, Jr.** 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature **418:**291.
24. **Leggett, D. S., J. Hanna, A. Borodovsky, B. Crosas, M. Schmidt, R. T. Baker, T. Walz, H. Ploegh, and D. Finley.** 2002. Multiple associated proteins regulate proteasome structure and function. Mol. Cell **10:**495–507.
25. **Liu, C. W., X. Li, D. Thompson, K. Wooding, T. L. Chang, Z. Tang, H. Yu, P. J. Thomas, and G. N. DeMartino.** 2006. ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome. Mol. Cell **24:**39–50.
26. **Mattson, M. P., and T. Magnus.** 2006. Ageing and neuronal vulnerability. Nat. Rev. Neurosci. **7:**278–294.
27. **Mayeux, R.** 2003. Epidemiology of neurodegeneration. Annu. Rev. Neurosci. **26:**81–104.
28. **McGuire, S. E., P. T. Le, A. J. Osborn, K. Matsumoto, and R. L. Davis.** 2003. Spatiotemporal rescue of memory dysfunction in *Drosophila*. Science **302:**1765–1768.
29. **Mizushima, N., B. Levine, A. M. Cuervo, and D. J. Klionsky.** 2008. Autophagy fights disease through cellular self-digestion. Nature **451:**1069–1075.
30. **Morimoto, R. I.** 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev. **22:**1427–1438.
31. **Morley, J. F., H. R. Brignull, J. J. Weyers, and R. I. Morimoto.** 2002. The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **99:**10417–10422.
32. **Taylor, J. P., J. Hardy, and K. H. Fischbeck.** 2002. Toxic proteins in neurodegenerative disease. Science **296:**1991–1995.
33. **Toba, G., T. Ohsako, N. Miyata, T. Ohtsuka, K. H. Seong, and T. Aigaki.** 1999. The gene search system. A method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster*. Genetics **151:**725–737.
34. **Tower, J., G. H. Karpen, N. Craig, and A. C. Spradling.** 1993. Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. Genetics **133:**347–359.
35. **Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates III, E. V. Koonin, and R. J. Deshaies.** 2002. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science **298:**611–615.
36. **Vernace, V. A., L. Arnaud, T. Schmidt-Glenewinkel, and M. E. Figueiredo-Pereira.** 2007. Aging perturbs 26S proteasome assembly in *Drosophila melanogaster*. FASEB J. **21:**2672–2682.
37. **Voges, D., P. Zwickl, and W. Baumeister.** 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68:**1015–1068.
38. **Walker, G. A., and G. J. Lithgow.** 2003. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. Aging Cell **2:**131–139.
39. **Warrick, J. M., H. L. Paulson, G. L. Gray-Board, Q. T. Bui, K. H. Fischbeck, R. N. Pittman, and N. M. Bonini.** 1998. Expanded polyglutamine protein

forms nuclear inclusions and causes neural degeneration in *Drosophila*. Cell **93:**939–949.
40. **Yamazaki, D., J. Horiuchi, Y. Nakagami, S. Nagano, T. Tamura, and M. Saitoe.** 2007. The *Drosophila* DCO mutation suppresses age-related memory impairment without affecting lifespan. Nat. Neurosci. **10:**478–484.
41. **Yao, T., and R. E. Cohen.** 2002. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature **419:**403–407.
42. **Yokoyama, K., K. Fukumoto, T. Murakami, S. Harada, R. Hosono, R. Wadhwa, Y. Mitsui, and S. Ohkuma.** 2002. Extended longevity of *Caenorhabditis elegans* by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. FEBS Lett. **516:**53–57.
43. **Yun, C., A. Stanhill, Y. Yang, Y. Zhang, C. M. Haynes, C. F. Xu, T. A. Neubert, A. Mor, M. R. Philips, and D. Ron.** 2008. Proteasomal adaptation to environmental stress links resistance to proteotoxicity with longevity in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **105:**7094–7099.